Evaluation of different staining techniques for determination of membrane status in spermatozoa

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ABSTRACT

Changes in the composition and integrity of spermatozoal membranes occur both during the normal capacitation and acrosomal reactions involved in the fertilization processes and as a result of different handling and storage procedures. Techniques to monitor these changes are essential to any study on methods of sperm preservation and such techniques should be both accurate, simple and rapid to apply.

A series of tests were conducted to evaluate a range of staining techniques to measure the integrity of the sperm plasma membrane (permeable membrane indicates non-viable sperm) and to measure the status of the acrosomal membrane (indication of capacitation and acrosomal reaction). The possibility of a combination of stains to provide simultaneous answers to both parameters was also attempted. Semen both fresh and frozen from both rams and bulls was used. The stains evaluated were: Nigrosin-Eosin (NE), Nigrosin-Eosin-Giemsa (NEG), Hoechst 33258 vital stain as both wet and dry mounts, Carboxyfluorescein and Propidium Iodide (CF-PI), SYBR-14 and Propidium Iodide (SYBR-PI), Pisum sativum -fluorescein isothiocynate (PSA-FITC), Chlortetracycline (CTC) and a FITC- labeled monoclonal-antibody (Mab) to an acrosomal protein. The techniques were graded for their accuracy, ease of use, equipment requirements and also for repeatability or adaptability to spermatozoa in different media. No single or combination of techniques proved suitable for both plasma and acrosomal membranes and most techniques varied in their suitability for different semen preparations.

Keywords: Spermatozoa; acrosome; viability stains; fluorescence microscopy.

INTRODUCTION

There is a need for accurate and rapid techniques to determine the viability and functional status of spermatozoa as part of the suite of laboratory tests required to provide an *in vitro* assessment of semen fertility. Such tests are also required to measure the changes that occur in spermatozoa during the process of collection, dilution, handling and storage prior to insemination. A measure of integrity of the spermatozoal plasma membrane is the exclusion of membrane impermeable dyes from the sperm cell. Such techniques are generally interpreted or classified as so called "live/dead" tests.

During the processes involved in *"in vivo"* fertilization the sperm cell undergoes considerable modification of the sperm head membranes, particularly the acrosomal membrane through the processes of capacitation and acrosomal excitosis (acrosome reaction).

These processes usually take place in the female tract and in close proximity to the ovulated oocyte for successful fertilization to occour. However changes in the acrosomal membrane similar to those of capacitation have been described as resulting from semen handling and storage especially as a result of the freeze/thaw process. Techniques to measure the changes in acrosomal status of sperm have depended on optical differences, contrast staining, lectin binding to surface molecules, monoclonal antibodies to acrosomal proteins and stains that reflect the changes in the distribution of calcium in the acrosomal and plasma membranes.

This paper reports on the results obtained with the evaluation of a range of different techniques to develop a reliable measure of sperm viability or function.

MATERIALS AND METHODS

The techniques evaluated are listed in Table 1 which also presents an indication of the equipment required to perform the evaluation.

All techniques were evaluated on both fresh (diluted) and frozen/thawed ram and bull semen, with a minimum of three different ejaculates of each type being tested. In general two separate aliquots of each semen type was tested

TABLE 1: List of Stains evaluated

Stain	Target	Equipment
Nigrosin + Eosin	L/D + Acrosome	Light-Microscopy.
Nigrosin + Eosin + Giemsa	L/D + Acrosome	Light Microscopy.
Hoeschst 33258	L/D + Acrosome	Fluor Microscopy.
Carboxyfluorescein + Propidium Iodide	L/D	Fluor Microscopy.
SYBR-14 + Propidium Iodide	L/D	Fluor Microscopy.
Pisum sativum - (FITC)	Acrosome	Fluor Microscopy.
Acrosomal protein Mab-(FITC)	Acrosome	Fluor Microscopy.
Chlortetracycline	Capacitation	Fluor Microscopy.

and after dilution a minimum of 200 cells in each of 2 drops were counted. Unless otherwise stated the fresh and frozen semen samples were diluted in RSD-1 (Upreti *et al.*, 1995). For the evaluation of the 'live-dead' stains a gradient of samples were prepared by taking half of a fresh semen sample and killing the sperm by rapid immersion into liquid nitrogen and rapid thawing (repeated 3x) and then mixing the fresh and killed sperm in different proportions. Changes in membrane status due to storage in different diluents, temperatures and exposure to chemicals known to induce changes in the acrosome of other species were investigated. Ram and bull sperm gave similar results and a selection of the evaluations performed with each technique using ram semen are presented in the results for purposes of illustration of the general findings.

Details of the staining techniques.

1. **Nigrosin + Eosin:** A stain made as described by Thompson & Cummins (1985) was used.

   Five µl of stain was added to 10 µl of semen at a concentration of (10-50 x 10^6/ml) on a slide, mixed and a smear made and allowed to air dry. A coverslip was mounted with DPX and observed under bright field at 400 x magnification (200 cells counted per slide in duplicate). Membrane intact cells are unstained while other cells are stained pinkish - purple.

2. **Nigrosin - Eosin - Giemsa:** (Tamuli & Watson, 1994). The N + E stain above was used and a 0.4% w/v stock solution of Giemsa (Sigma GS-500) was prepared. Five µl of semen (800 x 10^6/ml) and 15 µl N + E stain were mixed on slide, smears made and dried on a heated stage (37°C) for 30 min. Smears were fixed in PBS + 4% formaldehyde for 10 minutes, rinsed with H_2O for 7 min and immersed in Giemsa working solution for 60 min. Slides were rinsed in water and air dried. Sperm cells were examined under bright field at 1000x magnification under oil immersion. Dead sperm stain purple in the post-acrosomal region. Live sperm stain pink while the acrosomal region stains purple once the acrosomal reactions had commenced.

3. **Hoechst 33258:** This stain was used in both a dry and wet mount preparation with the latter technique claimed to enable acrosomal differentiation (de Leeuw *et al.*, 1991).

(a) Dry preparation: Fifty µl of of Hoechst 33258 (0.25 mg/ml) in citrate buffer was mixed with 50 µl of sperm at (10 x 10^6 per ml) and incubated for 5 minutes at room temperature. A 10 µl drop was placed onto a slide and gently smeared over an area of 2 cm^2, air dried and observed under epifluorescence using a Nikon UV-1A filter block - [excitation filter (EX=365nm), dichroic mirror (DM=400nm), barrier filter (BA=420nm)] at 500 x magnification.

(b) Wet Mount: Fifty µl of formaldehyde was mixed with 50 µl semen (10 x 10^6/ml), after 1 minute 250 µl of PBS solution was added. Fifty µl of this mixture was then mixed with 50 µl of Hoechst 33258 (0.5 mg/ml) and incubated for 5 minutes at room temperature. A 5 µl drop was placed on a slide covered with a coverslip and examined as described above.

4. **Carboxyfluorescein diacetate and propidium iodide:** The method of Harrison and Vickers (1990) was followed. Semen (10 x 10^6 /ml) mixed in equal volume (20µl) with staining material [paraformaldehyde (1.7 mM), 6 carboxyfluorescein diacetate (20 µM) and propidium iodine (7.3 µM)] and incubated for 8 min at 30°C. Then 8 µl aliquot placed on slide and overlaid with coverslip - examined under epifluorescence [Nikon B-2A - (EX/DM/BF = 450-490/510/520nm) and G-2A - (EX/DM/BF = 510-560/580/590nm) filter blocks both used in each field] at 400 x magnification. Intact sperm stain green while damaged sperm stain red.

5. **SYBR14 and propidium iodide:** For this stain a commercial kit (Molecular Probes "Fertilight" sperm viability kit - L-7011) was used according to the method described by Garner *et al.* (1994). Forty-five µl of semen (25-50 x 10^6/ml)was placed in an Eppendorf tube and 5 µl each of SYBR-14 in DMSO (1 µM) and PI (120 µM) in BSA-saline (1 mg/ml) was added and incubated at 37°C for 10 minutes. Three 10 µl drops of the mix placed on a slide under coverslips and evaluated under epifluorescence using Nikon B-2A filter block at 400 x magnification. Both live (green) and dead (red) sperm are seen simultaneously.

6. **Pisum sativum aggluten lectin (PSA) - conjugated to fluorescein isothiocynate (FITC):** The method used was based on that of Centola *et al* (1990). Frozen semen samples have to be cleaned of the freezing diluents (due to binding of stain to milk and egg yolk particles) using either sucrose or percoll gradients. Twenty-five µl of sperm suspension (10-20 x 10^6/ml) was carefully smeared onto a slide and allowed to dry. The sperm cell membranes were permeabilised by placing the slide into a 95% alcohol solution at 4°C for 15 minutes then removed and allowed to dry. PSA - FITC (Sigma L0770) solution (35µl of 0.1 mg/ml) was placed over the smear and incubated in a humidified chamber at 38°C for 15 min rinsed with PBS and allowed to dry. A coverslip was mounted using a drop of Dabco and sealed with nail varnish. Slides were examined under epifluoresence using a Nikon B-2A filter block at 400 or 1000x magnification.

7. **Mab stain:** A mouse monoclonal IgG antibody against a human acrosomal protein (50KD) prepared by Dr H. Moore, Sheffield University, was used with a fluorescence labeled second antibody (anti-mouse IgG - FITC, Sigma F9137). Twenty-five µl of sperm (10 x 10^6/ml) was carefully smeared onto a glass slide and allowed to dry. Sperm membranes were permeabilised by immersion in 95% alcohol at 4°C for 5 minutes and then dried. Mab solution (35 µl of a 1:100 dilution) was placed over the smear and incubated in a humidified chamber at 38°C for 15 min. The slide was rinsed with PBS solution and

allowed to dry and then 35 µl of the second-antibody (1:1000 dilution) placed on the smear and incubated for a further 15 min , rinsed and dried. A coverslip was mounted with Dabco and then sealed with nail varnish. Slides were examined using epifluorescence with Nikon B-2A filter block. Sperm with intact acrosomes fluoresced but non-stained sperm needed to be visualised under bright field illumination of the same field.

8. **Chlortetracycline (CTC) stain:** The method was based on that of Fraser *et al.* (1995). CTC solution (45 µl) and 45 µl of sperm preparation (250 x 10^6/ml) were mixed in an Eppendorf tube and 30 seconds later 20 µl of paraformaldehyde fixative (0.1%) was added at 37°C. Once fixed, tubes were stored in the dark. One drop of Dabco and 10 µl of stained sperm sample were placed on a slide and gently mixed and then covered with coverslip and sealed with nail varnish. Slides were examined under epifluorescence using a Nikon BV-2A - (EX/DM/BF = 400-440/455/ 470nm) filter block. Sperm with intact acrosomes showed an even bright yellow fluorescence over the entire head of the sperm. Capacitated sperm showed bright fluorescence in the acrosomal region only, while reacted sperm showed only slight staining of the head - sometimes with a brighter equatorial band. All sperm showed bright fluorescence of the mid-piece.

RESULTS

Table 2 provides an overall summary of the evaluations with the techniques being graded on the ease and time of preparation, the difficulty in diagnosis (ability to distinguish the different categories) and the time taken as well as the relative cost of the equipment required for the measurements.

Details of advantages and disadvantages:

1. **N + E:** Quick and easy to prepare but laborious and time-consuming to count. Preparation of smear and thickness can markedly influence results and cause difficulty in reading and possible bias due to selection of areas on the slide in which to perform the count. The presence of freezing diluents makes counting more difficult.
2. **N + E + G:** Same as for N + E but we also encountered the loss of sperm from slide during washing and failure of the Giemsa used to clearly define the acrosome. The staining time was longer and there was a discrepancy in the comparison of results obtained between the two stains when ram semen was stored in RSD-1 at 15°C for two or more days (Table 3) .
3. **Hoechst 33258:** Quick to prepare but had problems with reading due to a wide gradient of staining intensity that were hard to classify. In the wet mount the acrosomal status was not able to be distinguished under phase-contrast microscopy. Ram semen gave more problems than bull sperm and freezing diluents tended to distort the results.
4. **CFA + PI:** The CFA fluorescence quenched very quickly and while both the green and red could be seen simultaneously under blue excitation for a short period, the time needed to count adequate numbers meant that you had to alternate between the blue excitation (CFA) and green excitation (PI) on the same field on the slide. This added to the time required to perform counts. Variation in the patterns of staining (some sperm double stained) also influenced the categorization of sperm.
5. **SYBR-14 + PI:** Easy to prepare and quick to read due to bold and obvious staining.

Good correlation's between N + E and the CFDA + PI and SYBR-14 and PI methods were seen with the staining of gradient mixtures of fresh and killed sperm (Tables 4a and b).

TABLE 2: Overall summary of the evaluation

Stain	Preparation	Diagnosis	Equipment cost
N-E	Easy (batch variation.)	Difficult. (Subjective.)	Low
N-E-G	Long (batch var.)	Difficult. (Subjective.)	Low +
H33258	Easy	V. Difficult (ram)	Med-High
CF + PI	Easy	Difficult. (Subjective.)	Med
SYBR+PI	V. Easy	Easy	Med- V.High*
PSA-FITC	Easy (longer)	Easy	Med- V.High*
Acrosome-Mab	Easy (longer)	Medium	Med
CTC	Easy	Difficult. (Subjective.)	Med

* use of flow cytometer

TABLE 3: Comparison of N+E and N+ E+ G stains to measure the effect of storage at 15°C and incubation at 38°C on ram semen in RSD-1

Storage (days)	Incubation (h)	N+E (% live)	N+E+G (% live)
0	1	63	63
	24	67	69
1	1	65	69
	24	48	40
2	1	59	60
	24	38	64
7	1	28	58
	24	29	41

TABLE 4a: Comparison of N+E and CF+PI stains on ram semen

Gradient % fresh semen	CF+PI % live	N+E % live
100	85	87
75	57	58
50	45	45
25	22	23
0	0	3

TABLE 4b: Comparison of SYBR-14 + PI and N + E stains on ram semen

Gradient % fresh semen	SYBR-14+PI % live	N+E % live
100	67	69
75	51	50
50	35	38
25	18	19
0	0	0

6. **PSA-FITC:** Relatively easy to prepare and easy to read due to the bold staining. Smear preparation is critical and semen samples need to be cleared of the freezing diluents (especially egg yolk, milk etc) to avoid background fluorescence. Some variation found in the preparation for staining especially in the disruption of the acrosome and there is a need to develop best fit categories for classifications.
7. **Mab:** Smear preparation critical and also need for semen clean-up in frozen samples. Because the acrosomal reacted sperm did not fluorescence at all then it was necessary to view the same fields under bright field optics to count these and this adds to the time for counting.
8. **CTC:** there was a problem with fast quenching of fluorescence so it was necessary to add "anti-fade" solutions. The "wet mount" preparation results in some sperm with the incorrect orientation (not flat on slide) which cannot be classified. Sperm clean-up from freezing diluents is essential for this technique to work and also must use fresh preparations of stains each day which adds to the preparation time. Need examination at high magnification to put into "best fit" categories so counting can be slow. Also tendency of sperm to clump together after clean-up. However it is the only method that distinguishes capacitated and intact sperm. A comparison of the PSA and CTC stains on ram sperm exposed to different concentrations of Ca ionophore for 15 minutes to induce the acrosomal reaction is shown in Table 5, while the use of CTC to monitor the effect of sheep serum on the capacitation of fresh ram sperm (Crozet et. al. 1987) is shown in Table 6. It is important to note that nearly all frozen/thawed sperm show the capacitated CTC pattern within minutes of thawing.

General: Attempts to combine Hoescht 33258 or PI (to label dead cells) with either the PSA or CTC methods were not successful due to leaching of these stains following permeablisation and then being picked up by all cells.

TABLE 5: Comparison of PSA and CTC to measure acrosomal changes in ram sperm exposed to Ca Ionophore for 15 min.

Ca Ionophore. conc.	PSA		CTC		
	Reacted	Intact.	Reacted	Capacitated	Intact.
0.0 uM	12	88	16	29	55
0.3 uM	8	92	19	39	43
0.6 uM	52	48	34	22	44
1.0 uM	59	41	40	38	22

TABLE 6: Use of CTC to measure effect of sheep serum on capacitation of fresh ram semen

Time (h)	Diluent	Reacted	Capacitated.	Intact.
1.5	HSOF	4	5	91
	HSOF+20%SS	6	8	86
4	HSOF	15	7	78
	HSOF+20%SS	1	21	78
7	HSOF	20	19	61
	HSOF+20%SS	10	44	46
24	HSOF	30	19	51
	HSOF+20%SS	78	20	2

CONCLUSIONS

The results obtained from this series of evaluations has highlighted the variability in sample preparation and interpretation using light microscopy techniques and given the numbers of cells that need to be counted to obtain statistically reliable results (Smith *et al.* 1996) make them unsuitable for routine use with large numbers of semen samples. The use of fluorescence microscopy techniques using SYBR-14 + PI for viability and PSA-FITC for acrosomal status are recomended as rapid effective methods of evaluation. While more laborious the use of CTC remains the only choice to define capacitation. The problem of combining the acrosomal and vitality stains has since been resolved by a modification of the PSA/PI staining technique and the use of flow cytometry for counting (Ashworth *et al* 1995). This system has also been used for the SYBR-14 + PI method (Garner *et al.* 1995) and both flow methods are rapid to prepare and very rapid to read. Also because thousands of cells are counted in a very short time with this method the need for many duplicate readings to obtain a statistically meaningful value has been eliminated.

Studies are currently under way to provide information on the relationship between the results obtained on semen samples using these and other laboratory techniques and the *in vivo* fertility of that semen following insemination in the field.

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